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ABSTRACT

Many challenges arise when trying to amplify and analyze human samples collected in the field due to limitations in sample quantity, and contamination of the starting material. Tests such as DNA fingerprinting and mitochondrial typing require a certain sample size and are carried out in large volume reactions; in cases where insufficient sample is present whole genome amplification (WGA) can be used. WGA allows very small quantities of DNA to be amplified in a way that enables subsequent DNA-based tests to be performed. A limiting step to WGA is sample preparation. To minimize the necessary sample size, we have developed two modifications of WGA: the first allows for an increase in amplified product from small, nanoscale, purified samples with the use of carrier DNA while the second is a single-step method for cleaning and amplifying samples all in one column. Conventional DNA cleanup involves binding the DNA to silica, washing away impurities, and then releasing the DNA for subsequent testing. We have eliminated losses associated with incomplete sample release, thereby decreasing the required amount of starting template for DNA testing. Both techniques address the limitations of sample size by providing ample copies of genomic samples. Carrier DNA, included in our WGA reactions, can be used when amplifying samples with the standard purification method, or can be used in conjunction with our single-step DNA purification technique to potentially further decrease the amount of starting sample necessary for future forensic DNA-based assays.

Keywords: whole genome amplification, multiple displacement amplification, DNA purification, limited sample size

1. INTRODUCTION

1.1 Background

There are two major problems presented with regards to DNA purification and subsequent forensic analysis: 1) Forensic samples arrive in various physical forms such as hair, bone, blood, semen, vaginal fluids, skins cells, saliva, and tissue from which DNA must be isolated and purified. 2) The amount of DNA collected from the purification steps is always subject to a percent loss, and when the starting masses are small, the percent loss can be a significant proportion of template. Purified DNA is used for human typing and forensic analysis, which, depending on the assay and the amount of template collected, can exhaust the entire purified sample. Aside from limiting post clean-up analysis to a few forensic applications the percent loss can ultimately cause the purified sample to not satisfy the starting mass requirement for varying forensic techniques. Thus, the loss of purified DNA from clean-up can be compounded by the exhaustion of what little sample was available by a single forensic assay.

Whole genome amplification is able to bypass the constraints of limited starting material on carrying out post-purification assays. However, WGA is generally thought of only if insufficient amounts of DNA are present for all desired forms of analysis. If sample size is not a limiting factor, there are a number of processes that are effective in the field of forensics.

1.2 Alternatives to WGA

Many of the currently used technologies in forensic science require substantial amounts of starting template. Various techniques used for DNA typing and human sample identification are described here:

1.2.1 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism analysis was one of the original forensic techniques used to identify individuals in cases pertaining to paternity testing and other criminal investigations. Using larger quantities of high molecular weight genomic DNA, this technique is used to cut around specific regions of the human genomic DNA known to contain variable number tandem repeats (VNTR). Each region is flanked by a sequence of DNA universal throughout all humans that is a recognized cut site by specific endonucleases. The resulting fragments are then analyzed by electrophoresis or southern blotting. Although initially useful this technique is not used as frequently due to the large DNA template requirements (approximately 50 ng). [1] Also degraded samples do not work well either.

1.2.2 PCR analysis

PCR analysis is allele specific and can be used on cleaned genomic DNA. Based on the mechanics of PCR the initial DNA starting size necessary for amplification is theoretically 1 copy of DNA. However, reaction volumes, DNA target location by the polymerase and primers, and target size cause the actual starting amount to be higher. In order to determine the amount of purified DNA necessary for successful amplification, the sample quality (i.e. degradation and age), GC content, and size of desired product need to be taken into consideration. There are a number of different loci and alleles that are potential human identifiers, and the standard amount of starting template for each is from approximately 250 pg to 8 ng[1]. In some instances 250 pg may be the entire purified DNA sample, thus limiting other forensic applications and causing a lack of DNA for storage purposes.

1.2.3 STR analysis

Short tandem repeat (STR) profiles, or DNA fingerprints, can be generated for separate individuals by looking at specific loci of nuclear DNA. The FBI uses 13 STR regions per profile. But approximately 1 to 2 ng of DNA (~200 cells) is required (Promega GenePrint Fluorescent STR Systems Technical Manual, [1]) for STR analysis.

1.2.4 Mitochondrial DNA analysis

When RFLP and STR cannot be used, or when no nucleated cellular material is available (old biological samples like hair, bones), mitochondrial DNA analysis can be performed. In order to obtain optimal PCR results approximately 100 pg or more of extracted DNA should be amplified[1]. Products should also be sequenced.

1.2.5 Sequencing

DNA sequencing is a post-amplification method used to type the amplification products. Both STR PCR and mitochondrial PCR require this step for their analysis to be complete[1]. Thus, the limitations upon DNA sequencing are relative to minimum sample size required for STR and mitochondrial PCR to be carried out.

2. CURRENT STUDY

2.1 Amplifying small samples

When only smaller sample sizes are available for forensic analysis, WGA reactions are optimal. Increasing the chances of amplifying small samples can happen in two places: 1) when the small amount of DNA is already purified, the efficiency with which the DNA is amplified can be improved by using carrier DNA; and 2) when the limited DNA sample is contaminated, problems with sample recovery can be considerably reduced if the purification and amplification processes themselves occur within a single-use, packed column. Additionally, if these two methods are used in conjunction with one another, there is promise of lowering the needed amount of starting DNA for forensic applications even further.

2.2 Optimizing the amplification step

2.2.1 Choosing Φ29 polymerase

Eluted DNA samples are typically collected and then directly submitted for forensic analysis without pre-analysis Whole Genome Amplification (WGA). However, current interest in a thermostable and processive polymerase may change the limitations that sample size places on researchers as well as on the number of downstream applications applicable.

Over the past 20 years a heightened interest in Φ29 polymerase and its implications has generated a substantial body of literature describing the polymerase. Φ29 polymerase was discovered while studying Bacteriophage Φ29, found in *Bacillus subtillus*, and was determined to be the enzyme necessary for synthesis of the bacteriophage's linear DNA[2-5]. Φ29 has the ability to replicate a strand of DNA with one molecule, while having another molecule of polymerase displace the replicating strand hence the term Multiple Displacement Amplification (MDA)[6]. This method is highly efficient as well as processive. Blanco et.al found that the polymerase was capable of synthesizing up to 70,000 nucleotides without dissociating from the template[7], making it highly ideal for longer amplification times, and thus more product. The error rate of such high nucleotide placement was studied and found to be from $1x10^6$ to $1x10^7$, which is attributed to the polymerase's exonucleaic activity [8]. Studies performed both in vivo and in vitro demonstrated the enzyme's ability to be highly processive without the addition of accessory proteins [7] thus making it applicable for reactions other than for the amplification of its own DNA. The highly processive nature was then utilized to amplify large circular DNA, such as BAC's and plasmids in an isothermal process called Multiply-primed Rolling Circle Amplification (MPRCA)[9]. The isothermal reaction capabilities and the reproduction of strands of DNA in an exponential fashion made Φ29 polymerase a promising candidate for linear, dsDNA amplification.

Work originally done by Dean et. al[10] incorporated the use of random priming hexamers and demonstrated that ds Human Genomic DNA was capable of being amplified by the enzyme. His work was the basis for the REPLI-g kit, a commercially available amplification kit produced by QIAGEN (Valencia, CA), that is optimized for sample masses of 10 ng of genomic DNA and above (QIAGEN) although 1 ng of DNA may be sufficient if the quality of DNA is good. The REPLI-g kit is a robust assay that does not require thermal cycling and is completed in 16 hours. The genomic DNA yield is up to 40 µg per 50 µl reaction (REPLI-g manual) and varies from 1000 b up to 10 Kb.The kit uses an alkaline denaturation step as well as exonuclease resistant random priming hexamers and Φ29 polymerase. Although this kit is able to produce reliable and consistent data, its limiting factor is the necessary sample size of 10 ng of DNA, which is comparable to 1400 eukaryotic cells, an ample starting size. Whole cells can be lysed and amplified in this kit, but at a concentration of >600 cells/µl.

As previously described MDA can be employed to greatly increase the molecular copies of a desired sample. However, the other limiting factors within current MDA reactions must be addressed. There are two main components of the reaction to consider when working with significantly smaller starting samples: 1) The sample to volume ratio of the WGA reaction must be considered and 2) The potential exists for the template to adhere to non-biological surfaces. When starting with fg's of a sample in a 50 µl reaction the likelihood of all reagents in the reaction meeting in a timely matter is reduced. Thus, we decided to optimize our reactions to a much smaller volume of 11µl. New England Biolabs (Ipswich, MA) is a supplier of the pure Φ29 polymerase which also includes a 10x Buffer and 10 mg/ml BSA. The availability of the isolated polymerase allowed more freedom in the design of our protocol to tailor it to smaller sample sizes.

2.2.2 Using carrier DNA

In addition to reducing the reaction volume we also addressed the issue of crowding agents and the adhering of template to non-biological surfaces. The use of carrier DNA and crowding agents in MDA reactions were examined as a means to minimize the effect of these phenomena. Macromolecular crowding agents, such as glycerol and polyethylene glycol, have been shown to increase the efficiency of ligation reactions as well as decrease background noise in southern hybridizations when added [11] .Crowding agents also positively affect reactions with low starting samples with little or no adverse effects, making them promising candidates as additives to the MDA reaction [12, 13]. However, our preliminary tests indicated that the reaction was inhibited by the addition of glycerol .

Carrier DNA, such as salmon sperm DNA, was the next candidate examined for increasing product yield from small starting samples. Salmon sperm DNA has been used to reduce background noise in Flourescent In-Situ Hybridization (FISH). The reduction in background noise is thought to be caused by the carrier DNA's affinity for nonbiological surfaces, such as the sides of a microfuge tube[14]. Carrier DNA is added to the MDA reaction mixture prior to the template DNA to allow time for the coating of non-biological surfaces, such as the sides of a microfuge tube. Thus the non-specific binding of precious DNA template molecules can be eliminated. The nature of the random priming hexamers used in the MDA reaction allows them to bind to the carrier DNA as a template. Thus, we hypothesized that cross-linking salmon sperm DNA would make it less susceptible to hybridization and polymerization. It is also hypothesized that the carrier DNA can act as a space excluder, which potentially helps direct the primers and polymerase to the target DNA. Therefore, irradiated salmon sperm DNA was examined as an additive in the MDA reaction to effectively produce larger quantities of small starting samples.

2.3 Optimizing the purification step

2.3.1 Current purification methods and kits

The thorough purification of initial DNA samples is crucial to allow for efficient downstream applications like amplification. Columns have been manufactured commercially that remove such contaminants and amplification inhibitors. The challenge of finding an effective system of DNA purification that also balances cost-efficiency, provides for rapid throughput, and minimizes sample losses during the process is one that many commercial products have tried to address. QIAGEN provides kits for genomic DNA purification like the QIAamp DNA Mini Kit that can be used as a precursor to WGA using their REPLI-g Kit. The QIAamp kit at the very minimum requires 5000 cells (~35 ng DNA) to be loaded onto the spin column, in order to recover enough product for WGA applications. This limit on sample size is not favorable in the field of forensics, being that it is considerably larger than the amount of DNA lifted from a fingerprint. If smaller samples need to be purified with the QIAamp spin columns, a poly-A carrier is recommended to be loaded with the DNA that is to be purified. Though this might resolve the problem of loading smaller sample sizes when purifying, the poly-A carrier then raises the possibility of nonspecific amplification with the REPLI-g kit.

Other kits from different manufacturers also have unfavorable limitations on the amount of starting material. Sigma-Aldrich (St. Louis, MO) isolates pure high molecular weight DNA with their GenElute Mammalian Genomic DNA Miniprep Kit, which requires 2×10^6 cells or 25 mg of tissue to yield 25 μg and 30 μg of purified DNA, respectively. If the Sigma option of purification needs a starting amount of 2×10^6 cells for maximal efficiency, this kit would not be applicable to purifying small nanoscale sample sizes. Though commercial purification columns from QIAGEN, Sigma, and others offer functional methods for sample clean-up, limited numbers of template DNA molecules to be amplified cannot undergo the risk of incomplete sample release. Thus we hypothesized that sample purification should occur in the same column as WGA and decided to create our own packed column.

2.3.2 Bead substrate and DNA-binding salt choices

When purifying DNA, there are a number of factors that can be considered, including methods to efficiently bind the DNA to a substrate, to thoroughly wash away the impurities, and to elute the maximal amount of DNA possible from the clean-up column. Binding DNA can be dependent on the type of beads used to pack the purification column, the flow-rate at which the sample is administered, and any carriers or blocking agents used to facilitate binding and block unwanted impurities, among others. Blood samples, for instance, have high amounts of natural PCR inhibitors and low copy numbers of genomic material, making it very challenging to obtain a forensically applicable amount of DNA from traces of blood. By adding guanidine thiocyanate at a most favorable concentration of 2M when loading the sample onto the purification column, a majority of impurities are retained, resulting in more recovered DNA[15]. The use of a chaotropic salt like guanidine thiocyanate causes DNA, not lipids, proteins or other macromolecules, to bind well to silica surfaces, thus leading to the use of silica-coated beads within the purification column[16]. Bound DNA is immobilized upon the silica beads, which makes the DNA less prone to denaturation or degradation by DNA-degrading enzymes. Small amounts of available DNA already have better odds of surviving the purification process when efficient binding that focuses on maintaining native configurations occurs first. Purifying limited DNA samples and amplifying using Φ29 polymerase with the aforementioned benefits, when combined in a single-use packed column, would most minimize the chances of losing the low numbers of template DNA that are available in small samples.

3. CONCLUSION

We have been working in our lab to establish this working model of small sample purification and amplification prior to WGA for downstream applications in forensics, and manuscripts are in preparation.

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